A staining protocol for identifying secondary compounds in Myrtaceae[1]

Hernan A. Retamales[2] and Tanya Scharaschkin[2]

[2]School of Earth, Environmental and Biological Sciences, Queensland University of Technology. 2 George Street, Brisbane, Queensland 4001 Australia.

Email addresses: HAR: hernanalfonso.retamales@student.qut.edu.au

TS: t.scharaschkin@qut.edu.au

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[2]Author for correspondence: hernanalfonso.retamales@student.qut.edu.au

ABSTRACT

- *Premise of the study*: Here we propose a staining protocol using TBO and Ruthenium red in order to reliably identify secondary compounds in the leaves of some species of Myrtaceae.
- *Methods and results*: Leaves of 10 species representing 10 different genera of Myrtaceae were processed and stained using five different combinations of Ruthenium red and TBO. Optimal staining conditions were determined as 1 min of Ruthenium red (0.05% aqueous) and 45 sec of TBO (0.1% aqueous). Secondary compounds clearly identified under this treatment include mucilage in mesophyll, polyphenols in cuticle, lignin in fibers and xylem, tannins and carboxylated polysaccharides in epidermis and pectic substances in primary cell walls.
- *Conclusions*: Potential applications of this protocol include systematic, phytochemical and ecological investigations in Myrtaceae. It might be applicable to other plant families rich in secondary compounds and could be used as preliminary screening method for extraction of these elements.

Key words*:* anatomy; Myrtaceae; Ruthenium red; staining; Toluidine blue

INTRODUCTION

Ammoniated ruthenium oxychloride (Ruthenium red) and the thiazine metachromatic stain Toluidine blue (TBO) are regarded as two effective biological stains (Chaffey et al., 2002). Both reagents are often used in plant staining protocols involving hydration, staining and dehydration (Johansen, 1940; Ruzin, 1999). TBO has been widely used in plant histology to highlight diverse anatomical components such as lignified and non-lignified cell walls, nuclei, polyphenols, tannins, suberin and others (O'Brien et al., 1964; Crews et al., 2003; Perez-de-Luque et al., 2005). TBO is a cationic stain that binds to tissue anions and gives two main spectra of reaction, purple-pink and green-blue (Baker, 1966). Ruthenium red is a polycationic stain that also has applications for electron microscopy (Luft, 1964; Colombo and Rascio, 1977). However it also has important applications for light microscopy to stain aldehyde fixed mucopolysaccharides, calcium-dependent reactions and specific staining of pectic substances, mucilage and gums (Colombo and Rascio, 1977; Dierichs, 1979; Perez de-Luque et al., 2006). Improving the visual contrast of these reactions depends upon staining time, concentration and particular characteristics of the tissue, which might be species-dependent (Ruzin, 1999; Zhao et al., 2005). The visualization of these features can be optimized using efficient staining protocols that stain certain chemical compounds contained for these structures (Johansen, 1940; Cutler et al., 2008).

In the case of the family Myrtaceae, compounds such as mucilage, pectins and polyphenols are abundant in the leaf mesophyll (Wilson, 2011). Mucilage, pectins and other chemical secretions are regarded as taxonomically significant characters for the family (Schmid, 1980; Keating, 1984; da Silva et al., 2012). Although Myrtaceae is a large family of plants with ca. 5500 species (Wilson, 2011), anatomical studies of leaves are scarce and reports on secondary compounds are limited. A number of species in the family are rich in chemical

compounds with medicinal and biochemical activity (Wollenweber et al., 2000; Kytridis and Manetas, 2006). Nevertheless, pharmacological studies rely greatly in plant anatomy and more staining alternatives are needed in Myrtaceae.

Staining protocols used in Myrtaceae to date mainly involve Safranin O or some combination of Safranin O with Alcian blue, Astra blue or Fast green (Schmid, 1980; Gomes et al., 2009; Cardoso et al., 2009; Soh and Parnell, 2011). There are variety of studies regarding staining of plant tissues with Ruthenium red and TBO (Littlefield and Wilcoxson, 1962; Leiser, 1968; Western et al., 2001; Stpiczyńska and Davies, 2009). However, there are no published studies about optimization of staining procedures for a specific plant family or taxonomic group. Due to the presence of particular chemicals in the species of the family, an alternative staining protocol may improve the resolution of tissues in anatomical sections. Here we report an experiment using different combinations and duration of staining with Ruthenium red and TBO, so as to reliably identify secondary compounds in the family Myrtaceae.

METHODS AND RESULTS

Sampling, fixation and sectioning—

Leaves of Australian and South American Myrtaceae from different genera were collected from the natural habitat of the species. Species were selected from different genera in order to encompass a diversity of leaf structures. Details of taxa, location and collector numbers are provided in the Appendix 1. Voucher specimens are currently housed at BRI and will be deposited at EIF and SGO at a later date. Leaves were fixed in FAA for 24-48h depending upon whether species had soft or hard leaves. Composition of FAA (for 100 ml) was 90 ml of 50% Ethanol, 5 ml of Glacial acetic acid and 5 ml of Formalin 37-40% (Johansen, 1940). Fixed material was dehydrated through a graded ethanol series and embedded in paraffin wax

(Johansen, 1940; Ruzin, 1999). Transverse sections (5µm thickness) were cut using a Leica RM2245 rotary microtome.

Staining procedure—

Samples were deparaffinised with xylene, and then gradually hydrated through a decreasing alcoholic series (ethanol 100%, 90%, 70%, 50%, distilled water). Histochemical staining of sections was performed using a 0.1% (w/v) solution of Toluidine blue (TBO) (Amresco, Solon, Ohio, USA) in distilled water and 0.05% (w/v) of Ruthenium red (Sigma-Aldrich Co., Saint Louis, Missouri, USA) in distilled water following Jensen (1962). Samples were stained with one or both reagents for different periods of time according to five treatments, namely T1, T2, T3, T4 and T5 (Table 1). All specimens were subjected to these treatments (T1-T5) in order to determine optimal staining conditions that can be used to reliably identify anatomical characters across Myrtaceae. After staining, slides were dehydrated using an increasing ethanol series (50%, 70%, 90%, 100%, xylene) and mounted with DPX (Sigma-Aldrich Co., Saint Louis, Missouri, USA). The sections were observed using a Nikon SMZ 800 Stereo light microscope (Nikon eclipse 50i compound) and pictures were taken using the NIS Elements digital image analysis software (Nikon Instruments Inc., Amsterdam, Netherlands). Interpretation of colours from histochemical staining was based on O'Brien et al., (1964), Chaffei et al., (2002), Zhao et al., (2005) and Perez de-luque et al., (2006).

A total of 10 sections were stained per treatment for each species, which corresponds to ca. 500 sections. The entire staining experiment from deparaffinise to mounting, takes approximately two hours. Details of the staining protocol and cautionary comments are presented in the Appendix 2.

Results—

Optimal staining protocol—

Histochemical reactions in leaves were notably different depending upon treatment. Staining with Ruthenium red for one minute and counterstaining with TBO for 45 sec (T5) proved to be the most effective combination for differentiating secondary compounds based on colour (Table 2). T5 also showed to be the most consistent treatment of the experiment, staining secondary compounds with similar colours and contrast uniformly in all the species. Under this treatment, polyphenols, carboxylated polysaccharides, mucilage and pectins were clearly visible in different parts of the leaf (Fig. 1). The treatment T5 allowed a proper contrast between the cuticle (blue-green for polyphenolic compounds) and the epidermal cells of most of the species. Apparently, *Myrceugenia parvifolia* (DC.) Kausel and *Luma apiculata* (DC.) Burret do not present polyphenols in cuticle, as lacking blue staining in this structure (Fig. 1). Vascular bundles presented also better-defined elements using this treatment, showing clear differentiation between lignified secondary cell walls and non-lignified primary walls. Lignified vessels and fibres were stained blue-green with TBO, allowing excellent visual contrast. On the other hand, non-lignified primary cell walls in xylem, secondary phloem and non-vascular tissues were stained red with Ruthenium red, similarly to other studies in plants (Zhao et al., 2005; Perez de-luque et al., 2006). Positive staining with Ruthenium red was suitable for observing pectic substances in the middle lamella of non-lignified primary cell walls. Ruthenium red also allowed direct observation of mucilage in the mesophyll of most of the species.

Even though T5 had similar results through all the species in terms of secondary compounds, there are some taxa with special anatomical features stained differently. The palisade parenchyma cells of *Gossia floribunda* (A.J.Scott) N.Snow & Guymer, *Decaspermum humile*

(Sweet ex G. Don) A.J. Scott and *Eugenia reinwardtiana* (Blume) DC. were stained strongly and appear darker than those of other species (Fig. 1) indicating the presence of tannins and polysaccharides. The epidermal cells of some species (*G. floribunda*, *E. reinwardtiana*, *Ugni molinae* Turcz.) contain tannins (stained blue) and carboxylated polysaccharides (stained pink) whilst the epidermal cells of other species (*M. parvifolia*, *Syzygium australe* (J.C. Wendl. ex Link) B. Hyland, *Waterhousea floribunda* (F.Muell.) B.Hyland) lack these compounds. The phloem sieve tube members of the midrib in certain taxa (such as *Acmena smithii* (Poir.) Merr. & L.M. Perry) have a dark-staining content, potentially tannins, whilst *M. parvifolia*, *U. molinae* and *W. floribunda* give contrasting examples of taxa without tannins in phloem.

The species *L. apiculata* and *M. parvifolia* reacted somewhat differently to the treatment T5, showing a different pattern and intensity of colours. Histochemical staining revealed abundance of pectic substances and mucilage in the mesophyll of *L. apiculata*, with a predominance of red staining over blue compared to the other species when treated with T5. In the case of *M. parvifolia*, staining was slightly weaker than the other nine species; however anatomical elements and secondary compounds were clearly differentiated.

Comparison between treatments—

Treatment T1 showed similar results to T5 in terms of staining reaction, but some secondary compounds are not clearly visible with T1. Polyphenols (e.g. lignin) are stained red in the treatment T1, T2 and T4, without clear differentiation between the midrib fibres and the mesophyll cells due to weak reaction of TBO (Fig. 2). Similarly, xylem and phloem are not easily differentiable under T2, T3 and T4 (Fig. 3). On the other hand, T5 allowed the clear observation of lignin in fibres stained blue for this dye (Fig.2) and polyphenols in cuticle (Fig. 3). Although there are some differences if the lignin is fresh (*in situ*), polychromatic

staining with TBO is a reliable method to identify this compound when the staining time is optimal (O'Brien et al., 1964). Overstaining with Ruthenium red (T2, T4) produced homogeneous red staining through the samples without any coloured enhancement of secondary compounds excepting for mucilage and pectic substances (Fig. 2, Fig. 3). In the case of T4, the action of TBO might be neutralized by Ruthenium red, which is regarded as a stronger stain (Dierichs, 1979; Chaffei et al., 2002). Treatment T3 with TBO for 2 minutes resulted in blue overstaining without the optimal polychromatic reaction in tissues.

The combination that showed the best results (T5) might be proposed as an alternative protocol to existing ones involving different stains in Myrtaceae. Procedures involving Safranin O or combination of Safranin O with Alcian blue, Astra blue or Fast green have shown satisfactory results in anatomical studies on the family. However, there are no published studies supporting the use of these stains and their advantages. In addition to the quality of the enhancement of secondary compounds, the use of Ruthenium red and TBO has advantages in terms of time and safety. Safranin O is a regressive stain and needs between 2 and 24 hours to be effective and requires destaining in distilled water (Johansen, 1940; Ruzin, 1999). Safranin O also requires differentiation with picric acid, hydrochloric acid or tannic acid, regarded as unstable reagents (Ashbrook et al., 2003). On the other hand, the use of Ruthenium red and TBO do not require much time (2-3 minutes) or dangerous reagents.

CONCLUSION

In this paper we introduced a double staining protocol using Ruthenium red and TBO. We have evaluated a number of different staining treatments with these reagents in order to reliably differentiate secondary compounds in leaves of some species of Myrtaceae. The best combination was determined as one minute of Ruthenium red and 45 seconds of TBO (T5). Under this treatment, a number of secondary leaf compounds can be clearly identified:

polyphenols, mucilage, carboxylated polysaccharides and pectic substances. This procedure enhances the contrast of secondary compounds, which are visible in a wide range of colours (green-blue-red-pink). The applicability, safety and effectiveness are the main advantages of this protocol when compared to similar staining procedures used in the family. Other staining protocols used in Myrtaceae require more time and involve unstable reagents as hydrochloric acid, tannic acid or even explosive compounds such as picric acid. This protocol involves relatively few reagents and offers the option of adjusting and varying the duration at each stage of the process. This procedure might be alternative to commonly used staining protocols in Myrtaceae. Although the best results were obtained using a certain combination of stains, it is advisable to test the full procedure in order to detect differences in the results with other taxonomic groups. Identification of secondary compounds in leaves of Myrtaceae is highly important for systematic, phytochemical and ecological studies. This protocol could be used as a screening method for deeper study or extraction of these compounds.

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Tables

TABLE 1. Staining treatments applied in this study based on duration of staining.

	Duration of Staining (min)	
Treatment	Ruthenium red (0.05% w/v)	TBO (0.1% w/v)
T1	2	1
T2	2	0.5
T3	0	2
T4	2	0
T5	1	0.75

TABLE 2. Secondary compounds identified with each treatment for all the species based on the colour resulting from the staining process.

	Secondary compounds (colours)																			
	Mucilage (red-pink)					Polyphenols, tannins, lignin (blue-green)					Carboxylated polysaccharides (pink)					Pectic substances and some tannins (red)				
Taxa / Treatments	**1**	**2**	**3**	**4**	**5**	**1**	**2**	**3**	**4**	**5**	**1**	**2**	**3**	**4**	**5**	**1**	**2**	**3**	**4**	**5**
Acmena smithii	+	+	-	+	+	+	+	-	-	+	+	-	-	-	+	-	+	-	+	+
Gossia floribunda	+	+	-	+	+	+	-	-	-	+	+	+	-	+	+	+	+	-	+	+
Decaspermum humile	-	+	-	+	+	-	-	-	-	+	+	+	-	+	+	+	+	-	+	+
Eugenia reinwardtiana	-	+	-	+	+	-	-	-	-	+	+	+	-	+	+	-	+	-	+	+
Luma apiculata	+	+	-	+	+	+	-	-	-	+	+	-	-	-	+	+	+	-	+	+
Myrceugenia parvifolia	-	+	-	+	+	+	-	-	-	+	+	+	-	-	+	-	+	-	+	+
Myrteola nummularia	-	+	-	+	+	+	-	-	-	+	+	+	-	-	+	-	+	-	+	+
Syzygium australe	-	+	-	+	+	+	+	-	-	+	+	+	-	-	+	-	+	-	+	+
Ugni molinae	+	+	-	+	+	+	+	-	-	+	+	-	-	+	+	-	+	-	+	+
Waterhousea floribunda	+	+	-	+	+	+	+	-	-	+	+	-	-	-	+	-	+	-	+	+

(+) Positive staining of secondary compounds

(-) No staining of secondary compounds

Figures and Legend

Figure 1. Transverse light micrographs (LM) of leaves of some species of Myrtaceae stained with the staining treatment T5. A, *Acmena smithii*: Clear highlight of lignified cells (blue) and mucilage (red). B, *Eugenia reinwardtiana*: Carboxylated polysaccharides in epidermis. C, *Syzygium australe*: Phloem cells stained red and xylem blue-green. D, *Gossia floribunda*: Mesophyll cells containing dark stained pigments, probably tannins. E, *Myrceugenia parvifolia*: Pectic substances in primary cell walls observed in spongy parenchyma (red). F, *Waterhousea floribunda*: Cuticle with polyphenols stained blue and fibres with lignin stained blue-purple. G, *Luma apiculata*: Red staining of pectic substances and mucilage. H, *Ugni molinae*: Mucilage in spongy parenchyma and clear difference between polyphenols in xylem (lignin) and phloem. Scale bars = 100 μm.

Figure 2. Transverse light micrographs (LM) of leaves of *Acmena smithii*, showing comparisons between treatments T2 (A-B), T3 (C-D), T4 (E-F) and T5 (G-H). Treatment T1 showed similar results to T5. A-B, Polyphenols highlighted in cuticle (blue) and mucilage (red), however lignified elements in midrib are not differentiated. C-D, Secondary compounds are not differentiated for masking of TBO. E-F, Secondary compounds are not differentiated for masking of Ruthenium red. G-H, Secondary compounds clearly visible in T5. Polyphenols in cuticle, xylem and fibers (lignin, blue), mucilage and pectic substances in mesophyll (red), tannins in phloem (red) and carboxylated polysaccharides in epidermis (pink). Scale bars = 100 μm.

Figure 3. Transverse light micrographs (LM) of leaves of *Eugenia reinwardtiana*, showing comparisons between treatments T2 (A-B), T3 (C-D), T4 (E-F) and T5 (G-H). Treatment T1 showed similar results to T5. A-B, Only polyphenols in cuticle (blue) are differentiated as the red staining is predominant. C-D, Secondary compounds are not differentiated for masking of TBO, except for the cuticle. E-F, Secondary compounds are not differentiated for masking of Ruthenium red. G-H, Secondary compounds clearly visible in T5. Clear difference between xylem and phloem. Polyphenols in cuticle, xylem and fibers (lignin, blue), mucilage and pectic substances in mesophyll (red), tannins in phloem (red) and carboxylated polysaccharides in epidermis (pink). Red tannins in epidermis observable with all the treatments. Scale bars = 100 μm.

Appendix

APPENDIX 1. Taxa, vouchers, geographic locality and GPS coordinates of samples collected for this study—

Taxon	Collection number*	Collection locality	Geographic coordinates
Acmena smithii (Poir.) Merr. & L.M. Perry	Reta-032.1-Reta-032.2	Robina, QLD, Australia	28° 4' 46" S / 153° 23' 17" E
Gossia floribunda (A.J.Scott) N.Snow & Guymer	Reta-031.1-Reta-031.2	Cooroibah, QLD, Australia	26° 21' 44" S / 152° 59' 3" E
Decaspermum humile (Sweet ex G. Don) A.J. Scott	Reta-029.1-Reta-029.2	Enoggera, QLD, Australia	27° 25' 21" S / 152° 59' 26" E
Eugenia reinwardtiana (Blume) DC.	Reta-028.1-Reta-028.2	Robina, QLD, Australia	28° 4' 46" S / 153° 23' 17" E
Luma apiculata (DC.) Burret	Reta-026.1-Reta-026.2	Osorno, Chile	40° 34' 0" S / 73° 9' 0" W
Myrceugenia parvifolia (DC.) Kausel	Reta-021.1-Reta-021.2	Puerto Montt, Chile	41° 28' 18" S / 72° 56' 12" W
Myrteola nummularia (Poir.) O. Berg	Reta-03.1-Reta-03.2	Futrono, Chile	40° 7' 28" S / 72° 22' 51" W
Syzygium australe (J.C. Wendl. ex Link) B. Hyland	Reta-034.1-Reta-034.2	Kawana Island, QLD, Australia	26° 43' 11" S / 153° 7' 41" E
Ugni molinae Turcz.	Reta-04.1-Reta-04.2	Talcahuano, Chile	36° 43' 0" S / 73° 7' 0" W
Waterhousea floribunda (F.Muell.) B.Hyland	Reta-030.1-Reta-030.2	Enoggera, QLD, Australia	27° 25' 21" S / 152° 59' 26" E

*Housed at BRI (Queensland Herbarium, Brisbane, Australia) and will be deposited at SGO (National Museum of National History, Chile) and EIF (Forestry Sciences Herbarium, University of Chile).

Appendix 2. Description of the protocol and troubleshooting—

Chemicals (vendors) used in this study

Xylene (Ajax Finechem Pty, Taren Point, New South Wales, Australia) CAS: 1330-20-7

Ethanol (Chem-Supply Pty, Gillman, South Australia, Australia) CAS: 64-17-5

Ruthenium red powder (Sigma-Aldrich Co., Saint Louis, Missouri, USA). CAS: 11103-72-3

Toluidine blue powder (TBO) (Amresco Co., Solon, Ohio, USA). CAS: 92-31-9

DPX (Sigma-Aldrich Co., Saint Louis, Missouri, USA). CAS: 14208-10-7

Distilled water

Equipment

Glass staining dishes with glass lids

Slide racks

Trays for drying slides

Paper towels

Fume hood

NitriSolve flock-lined gloves are recommended when handling xylene, and nitrile gloves for all other reagents.

Safety goggles

Microscope slides

Coverslips (24x24mm) and long coverslips (24x50mm)

Dropper bottle

Forceps, spoon, spatula

Preparation of staining solutions

Ruthenium red (Jensen, 1962)

1. Dissolve 0.3 g of Ruthenium red powder in 100 ml of distilled water.

2. Stir for 15-20 minutes until dissolved, without heat, until completely dissolved

3. Filter after stirring

4. Store in Schott bottle, refrigerated to prevent growth of microorganisms

Freshly prepared solution should look black in colour.

Toluidine blue

1. Dissolve 0.1 g of Toluidine blue in 100 ml of distilled water.

2. Stir gently

3. Filter

4. Store in Schott bottle, refrigerated to prevent growth of microorganisms

Histochemical staining procedure protocol

1. Place 20 staining dishes in a fumehood. Fill the dishes with the following solutions: 2x ethanol 50%, 2x ethanol 70%, 2x ethanol 90%, 2x ethanol 100%, 4x xylene, 1x xylene:ethanol (1:3), 5x distilled water, 1x aqueous solution of Ruthenium red and 1x aqueous solution of Toluidine blue.

2. Place slides with paraffin sections in slide racks and transfer to different solutions and stains using the sequence and duration outlined below:

Step	Solution	Purpose	Duration (estimated intervals)
1-2	Xylene	Deparaffinisation	15 min (two changes)
3	Xylene:ethanol	Hydration	10 min
4	Ethanol 100%	Hydration	5 min
5	Ethanol 90%	Hydration	5 min
6	Ethanol 70%	Hydration	5 min
7	Ethanol 50%	Hydration	5 min
8	Distilled water	Hydration	5 min
9	Ruthenium red	Staining	Variable
10	Distilled water	Removal of excess stain	30 sec
11	Distilled water	Removal of excess stain	30 sec
12	Toluidine blue	Counter staining	Variable
13	Distilled water	Removal of excess stain	30 sec

14	Distilled water	Removal of excess stain	30 sec
15	Ethanol 50%	Dehydration	5 min
16	Ethanol 70%	Dehydration	5 min
17	Ethanol 90%	Dehydration	5 min
18	Ethanol 100%	Dehydration	5 min
19-20	Xylene	Drying	10 min (two changes)

3. Check staining result under a microscope after step 14 before proceeding with dehydration. Adjust staining time if needed.

4. Mount and coverslip with DPX (Entellan®)

5. Lay coverslips out on a sheet of blotting paper and wipe clean to remove dust. Place an elongated drop of mountant (DPX) across the middle of each coverslip. Remove a slide from the xylene (Step 20 above), drain off the excess liquid and lay it, section side down, over the coverslip. Repeat for each slide, making sure that air bubbles are not trapped beneath the coverslip. If bubbles are present, press them gently towards the edge of the coverslip with a mounted needle. Wipe around the slide with a tissue to remove excess xylene or mountant. Dry the slides on a flat surface in the fume hood until the mountant has hardened sufficiently to allow handling.